IV. BIOAUTOMATICS AND BIOINFORMATICS

GROWTH MODELS IN MICROBIAL ECOSYSTEMS - RESOURCE OR DENSITY DEPENDENCE?

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Abstract. This paper aims at discussing the two main modeling schemes that are used to describe dynamically the growth of microbial ecosystems, that are resource and density-dependent growth functions, respectively. Monod has been the first to hypothesize that this growth is, before all, an increasing saturated function of the main limiting substrate concentration. Contois assumed that the growth is not only a function of the substrate but also of the biomass density-itself, and thus the name « density-dependent ». In re examining their respective experiments (species used, conditions of experiments, mode of reactor functioning, measurement techniques), we try to understand the engines for a density-dependent phenomenon to appear. In particular, we refer to recent experiments where it was shown that density-dependent appeared as soon as the biomass structures into flocs or in the presence of filamentous bacteria even at relatively low concentrations. Based on this historical review of data, it is shown that density-dependent kinetics is not systematically a question of biomass density but rather related to its structure within the medium and to the mobility of microbial cells.

Keywords: modeling growth rate, Monod, Contois, microbial ecosystems, microbial interactions, mathematical ecology.

1. INTRODUCTION

Modeling growth rates of a microbial ecosystem using either resource or density-dependent function is an old question. The classical resource-dependent function is given by the Monod function (Monod, 1949), written as:

$$\mu(S) = \mu_{max} \frac{S}{S + K_S}$$

while the classical Contois function (Contois, 1959) describes a density-dependent growth rate:

$$\mu(S, X) = \mu_{max} \frac{S}{S + K_C X}$$

In Jost, 2000, it is underlined that at the origin kinetics of pure cultures were rather modeled using Monod functions while growth kinetics of complex microbial ecosystems were rather modeled using Contois functions. The complexity of the models increasing over time, the additional coupling (between the substrate and biomass concentrations) imposed by the use of the Contois function gradually led to a more common use of the Monod function. Today, the most common models of biotechnology all use combinations of Monod functions and almost never the Contois function. It is for instance the case of models of the International Water Association successfully used to model complex wastewater treatment plants, *cf.* for instance IWA TG on ASM modeling, 2000. In his paper, Jost also insisted on the fact that such models were developed in parallel in different

scientific files, notable microbiology and ecology – with different names! While the «Monod function» is used in biotechnology and microbiology, ecologists rather term this model «Holling Type II». Likewise, while microbiologists use the term «Contois function», ecologists call the usual density-dependent function the «Arditi-Ginzburg functional response».

In literature, there exists hundreds of growth functions each proposed for specific culture conditions and species. Some authors have tried to review the available expressions as in the well know book by Bastin and Dochain, *cf.* Bastin and Dochain, 1990. What is important to understand here is that the main characteristics of a biological system, at the population scale, may globally be captured in usual functions such as Monod or Contois functions without complicating too much even if specific processes such as inhibition may also be incorporated in models.

In this paper, we first reexamine the experimental conditions used by Monod and Contois in their respective work. Then, we investigate which experimental conditions can likely explain the fact that the biomass growth is better modeled by one or the other function. We conclude that more important than the density of the biomass in itself, it could be the structure of this biomass, notably with respect to its mobility ability, which gives its density-dependent characteristic to a biological microbial ecosystem.

2. REVISITING MONOD AND CONTOIS EXPERIMENTS

In this section, we review the experimental work by Monod and Contois respectively. The data reported are taken from Monod's thesis and for Contois from his 1959 paper.

2.1. Biological material

2.1.1. Monod's works

For his experiments, Monod used two different bacterial strains.

Colibacillus or *Escherichia coli* is a gram negative bacterial genus with a single species, counting more than 1000 antigenic types, discovered by Thomas Escherich in 1855. The size of these bacteria which are not encapsulated is of the order of 0.5 to 1-3 microns. Usually isolated, they can group together in pairs or in a short chain. With a peritrichous flagellum, some have the possibility of moving. *coli* are facultative aerobes and have the ability (i) to ferment glucose, mannitol and lactose; (ii) reduce nitrates to nitrites; (iii) ferment the sucrose to salicin. Finally, they degrade tryptophan into indole. Its optimal environmental conditions for growth are: (i) neutral pH (7 to 7.5); (ii) a temperature of around 35°C. Under such conditions, the doubling time is about 20 minutes.

Bacillus subtilis is a rectilinear (or practically rectilinear) bacillus, with square or rounded ends, of variable size (from 0.7-0.8 to 2-3 μm), sporulated, Gram positive or Gram variable (frequently, the Gram is positive only in very young cultures, which means that the wall of old cells is fragile or at least permeable to alcohol), generally mobile thanks to a peritrichous ciliature. *B. subtilis* is part of Bacillus group IB comprising bacilli less than 1 μm in diameter and free of poly-beta-hydroxybutyrate inclusions. *B. subtilis* has been isolated in humans from endocarditis, pneumonia, bacteremia, sepsis, and eye infections. It is therefore an opportunistic pathogen like *E. coli*. This germ has been implicated in abortions in sheep and, in cattle, it is the agent of acute mastitis, recurrent despite antibiotic treatment (penicillin for example) which, in view of the results obtained in vitro, should have been effective. Its optimum growth temperature is between 30 and 37°C and is optional aerobic.

2.1.2. Contois's works

Contois, meanwhile, only used one microbial strain

Aerobacter aerogenes is a Gram-negative, non-motile bacteria, often encapsulated and usually

isolated. Their size is in the range of 0.5-0.8 to 1-2 microns. This species, discovered in 1885 by Escherich, is part of the *Aerobacter* genus characterized by their ability to ferment glycerol with the production of acid and gas. *A. aerogenes* also has the property of reducing nitrates to nitrites, using citric acid and citric acid salt as a source of nitrogen and uric acid as a source of nitrogen. These are facultative aerobic bacteria with maximum growth at temperatures around 30°C where their doubling time is then faster than for *E. coli*. In contrast, *A. aerogenes* is found to be more sensitive to higher temperatures since none can withstand 30 minutes at 60°C.

2.1.3. Conclusions on the biological material used

E. coli and *A. aerogenes* are two species belonging to the Enterobacteriaceae, a family characterized by a Gram-negative stain and their ability to reduce nitrates to nitrites (this corresponds to alternative anaerobic respiration during oxygen deficiency of the medium, *i.e.* a rescue breathing). This family includes five tribes including those of the Escherischeae having the property of fermenting lactose with the formation of acid and gas. This family has three genera, including Escherichea, to which *E. coli* belongs and which has the particularity of not producing acetylmethylcarbinol and of being negative on the methyl red test. The Escherischeae also include the genus *Aerobacter* to which *A. aerogenes* belongs which, unlike Escherichea, produce acetylmethylcarbinol and are methylene red positive.

So these two species are relatively close and there seems to be no contradiction in comparing or assimilating the data collected by Monod and Contois during their various works.

B. subtilis belongs to the Bacillaceae family, which has the property of producing spores and having ellipsoidal endospores located in the center of the cell. This family includes two genera, including Bacillus, to which *B. subtilis* belongs, which has the property of being aerobic, mesophilic and catalase positive. Nevertheless, Bacillus remains an ill-defined genus in the classification and in particular for *B. subtilis*. Indeed, there are different subspecies such as *B. subtilis* var. *aterrimus*, var. *niger*, var. *niger comb...* Also, the data collected by Monod with this species will be difficult to assimilate to those of *E. coli* and *A. aerogenes* because of the imprecision as to the precise strain used and its physiological properties.

2.2. Cultures conditions.

2.2.1. Monod's works

Monod's data were obtained from experiments in which specific growth rates were measured early in the cycle under batch culture conditions with different concentrations of limiting substrate. The cultures were carried out in Erlenmeyer flasks of 1 or 2 liters depending on the quantities of medium used. The temperature is kept constant at 37°C by a water thermostat of the Warburg type.

Aeration and mixing were ensured in two ways, depending on the case:

- by using a device that continuously agitated the flasks within the thermostat (this is an identical device to that used for Warburg manometers);
 - by injecting air directly into the medium.
- The air, from a compressed air cylinder, passed through a large glass ball filled with sterile cotton wool and entered the Erlenmeyer flask through a tubing terminated by a glass bubbler providing extremely fine bubbles.

During its experiments, Monod used two types of sterilized media:

- Natural media, consisting of broths from the brains of calves or sheep, which have the advantage of being almost colorless and make it possible to limit errors during measurements of population density by optical density;
- Synthetic media with glucose as a carbon source. As before, this medium never becomes cloudy during growth.

In both cases, the pH appears to be extremely stable and close to neutrality, hence the lack of a regulation system for this parameter.

2.2.2. Contois's works

Since Monod invented the continuous culture and that the pre described experiments were not realized in chemostats, it is quite funny to say that unlike the Monod study described above, Contois worked under continuous culture conditions. He was inspired for this by the experimental protocol developed by Novick and Szilard (1950).

The total volume of each reactor is between 30 and 45 ml and remains constant throughout the duration of the experiments, the feed and outlet flow rates being equal.

Aeration and agitation of the medium were ensured in each reactor by injection of sterile air at

the rate of 10 liters of air/ml of culture/hour as well as by rotating discs of 8 mm in diameter located at the bottom of the container.

The pH was not regulated during growth but all media were adjusted to 6.8 before inoculation.

Aerobacter aerogenes was cultivated by Contois on a synthetic salt medium comprising ammonium as a source of nitrogen and glucose or succinate as a source of carbon. Either of these two components is provided in limited quantities while all other nutrients are present in excess.

2.2.3. Conclusions on the conditions of culture used

Although Monod and Contois used different culture techniques (batch vs continuous), it is conceivable that this does not affect the comparison of their data. Indeed, the two authors carried out their measurements during the exponential growth phase, about which it can be claimed that it does not vary significantly between these two cultivation techniques.

It should also be noted that Monod and Contois have experimented with relatively low concentrations of carbon source material. This is, at most, 200 mg for Monod and 500 mg for Contois (as a comparison, a classical industrial wastewater may contain more than 10-20 g.l⁻¹ of carbon, measured as the COD).

With such low resource concentrations and total volumes of the order of a liter, it is obvious that the physical density of the population in relation to the available space (carrying capacity of the environment) has never been able to reach a sufficient value to constitute a limiting factor of growth!

2.3. Measurements

2.3.1. Monod's works

Monod carried out density measurements using a Meunier nephelometer (see figure 1) using blue light (wavelength not specified). Monod carried out several preliminary experiments in order to ensure (i) that the graduation read on the device was indeed proportional to the concentration of the bacterial suspension, (ii) that the ratio between the arbitrary units of the nephelometer and the absolute units of reference (dry substance weight) did not vary significantly during growth.

Fig. 1: Principle of meunier nephelometer used by Monod

As for *coli*, 10 optical density units corresponded to 7.9 mg.l^{-1} of dry matter per liter of culture in the exponential phase and 8 mg.l^{-1} at the time of the maximum. For *B. subtilis*, these values were respectively 7.6 and 7.5 mg.l^{-1} . Monod felt that there were no significant differences between these two values (although he did not run a statistical test to support this). He therefore concluded that the variation in the size of bacteria during growth cannot lead to a noticeable variation in the absolute value of the units of optical density (OD). Preferring to keep the data in OD units for his analyzes, he made the choice, when transformation into weight unit was necessary, to round the transformation ratio to 0.8 for the two bacterial species.

2.3.2. Contois's works

Population density was assessed through optical density using a spectrophotometer. Unlike the Meunier nephelometer which measures the light

scattered by a suspension, the spectrophotometer measures the light transmitted by it.

The optical density of the samples was measured in small 10 cm Pyrex cuvettes at a wavelength of 420 mμ.

Contois made comparisons between the OD values and those obtained by direct counting. Thus, he determined that one unit of optical density corresponded to 5×10^8 organisms/ml (the equivalence by weight of dry substance was unfortunately not appreciated).

2.3.3. Conclusions on the monitoring device used

The two authors therefore used optical density in order to estimate the density of the population and thereby derive growth rate values from it. However, their measurement methods (nephelometer vs spectrophotometer) differed. It is therefore not possible to directly compare the optical density values they obtained. It is necessary to convert this physical data into biological data. The results will therefore be expressed as the number of bacteria per liter and the limiting substrate concentrations in mM/l.

Monod estimated that 10 UO corresponded to approximately 8 mg.l^{-1} of dry matter. Considering that the dry mass of a bacterium is of the order of 2.8×10^{-13} grams for *E. coli* (Neidhardt and Umbarger, 2002), it appears that this author worked with concentrations of organisms between 2 and 20×10^{10} bacteria/l during its experiments for limiting substrate concentrations of 0.14 to 1.11 mM/l (see Table 1).

Table 1: Comparative table of the experimental data of Monod and Contois –
(i) Concentrations of limiting substrate (glucose) in mM/l, (ii) optical densities measured in UO,
(iii) Concentrations of microorganisms expressed as number of bacteria $\times 10^{10}$ /l.

	<i>[glucose] in mM/l in the medium</i>	<i>Measured optical densities (UO)</i>	<i>Number of individuals per liter ($\times 10^{10}$)</i>
Monod	0.14	6.7	1.93
	0.28	14.4	4.11
	0.39	20.0	5.71
	0.50	26.0	7.43
	0.67	35	10
	0.78	41.3	11.8
	0.89	46.8	13.4
	1.00	53	15.1
	1.11	58.7	16.8
Contois	1.07	0.49	0.245
	4.02	1.83	0.915
	4.84	2.2	1.10
	5.21	2.37	1.19
	5.3	2.4	1.20
	5.55	2.52	1.26

Contois, meanwhile, estimated that one UO corresponds to about 5×10^8 bacteria per liter. The latter worked with microorganism concentrations between 0.25 and 1.26×10^{10} bacteria/l for a limiting substrate concentration ranging from 1 to 5.55 mM/l

Thus, it should be noted that although Contois used limiting substrate concentrations up to five times higher than those of Monod, he was in the presence of microorganism concentrations lower than the latter, up to about 14 times less (cf. Table 1).

3. DISCUSSION

Monod function assumes the growth rate of microorganisms does only depend on the main limiting resource. The analysis of a one step biological system in a chemostat where the microbial population – named the biomass X – grows on a limiting resource – the substrate S – is well known. In particular, as long as the hydraulic retention time and the input substrate concentration are large enough, there exists a unique steady state $\{S^*, X^*\}$ which has the following characteristics: $S^* = \mu^{-1}(D)$ and $X^* = S_{in} - S^*$ assuming the yield coefficient equals 1, cf. Smith and Waltman, 1991 or Harmand *et al*, 2017. Contois has the same expression that Monod's equation but the argument is not S but S/X . In other terms, Contois equation does not only depends on the resource concentration but also on the biomass density. In more precise terms, it depends on the ratio between the available resource and the biomass present. The main argument to be taken into account is the fact that the rate decreases when the biomass increases. The main characteristic is that the ratio of resource and biomass concentrations at steady state is constant and depends on the hydraulic retention time. Fundamentally, with respect to single biological reactions in which a population grows on a single limiting substrate, using any of these functions does not change so much the issues of the predictions. However, it may lead to significant differences when considering complex systems, in particular when studying questions related to microbial diversity – several microbial species growing on the same limiting resource. In such a case, the asymptotic behavior of the system differs completely: the system with Monod growth rates predicts the survival of only the species that has the best affinity with the substrate while the system with Contois growth rate eventually enables the coexistence of all species, cf. cf. Smith and Waltman, 1991 or Harmand *et al*, 2017. It is the formalization within

the microbial world of the well known *Competitive Exclusion Principle* or CEP. Thus, in particular with respect to *complex biological systems*, the question of characterizing the laws that condition growth is very important. It is why several authors have proposed several experiments – mobilizing either what could be called *qualitative* or *quantitative* approaches to decide whether, and under which conditions, density-dependence appears in microbial growth, cf. Arditi and Saiah, 1992, Harmand and Godon, 2007 or still Krichen *et al.*, 2017. Here, we call *qualitative* an approach in which the conclusions are made without having to develop a specific model of the system. On the converse, we call it *quantitative* (or *heuristic*) when a specific model is developed. In particular, the approach proposed by Arditi and Saiah or a number of reasonings used in their book and only based on macroscopic observations realized on many ecosystems are typically qualitative while the approach followed by Krichen *et al.*, 2017 is typically quantitative.

But what may a *complex biological system* means? A biological system, as a living entity, could be said to be intrinsically *complex*. Here, we restrict the use of this term to situations where the main characteristics of an ideal chemostat are about to vanish. For instance, in the previous case, complexity refers to the presence of several species instead of only one in the system. In his paper, Jost recalled that complex microbial ecosystems such as those used for wastewater treatment plants – in the sense they are very diverse and in which biomass structures naturally in flocs – were likely modeled using density-dependent kinetics. When considering only one species, notice that as long as the biomass concentration is not too high, Monod and Contois functions will not differ so much. Significant differences arise when the biomass concentration becomes significantly high. As it continues to increase, the hypothesis of a perfect mixture of the medium may not be verified any more: the explanation that comes to our mind is that it becomes more difficult for any individual to access its substrate – the competition between individual increases – and this heterogeneity must be taken into account. It is precisely what a Contois function allows. As underlined hereabove, if several species are present, Contois function eventually enables the survival of all of them: one of the mathematical conditions to be verified for this to happen is that the intraspecific competitions be greater than the interspecific competition. And it probably happens: as microorganisms multiply by division, individuals

of the same species are more likely to be found next to each other, and thus are more likely in competition with individuals of the same species than themselves than with others. If the CEP has been tested – and underlined – in a (small) number of real experiments – putting in play a very limited number of species and in very controlled laboratory conditions – it is the rule rather than the exception to observe the maintenance of a high diversity in most real microbial ecosystems. We can conclude that in most real conditions, density-dependent is more likely found. If a number of explanations have been put forward, the main phenomenon at the origin of a density-dependent growth still remains obscure.

As suggested in the recent Arditi and Ginzburg book «How Species Interact: Altering the Standard View on Trophic Ecology», the main phenomenon could simply be the density in itself. In that, the intuitive reasoning when considering high concentrated microbial cultures is intuitively satisfying. And it would be particularly satisfying to come to these conclusions on the basis of Monod and Contois experiments. However, it is not the case at all! As reported in the section 2.3.3, both Monod and Contois worked with very low limiting resource concentrations. And most important than all, Monod seems to have worked with significantly higher biomass densities than Contois!

If the high density of biomass can obviously explain the occurrence of density dependence phenomena, it seems that it is not the only one, and in any case not the predominant phenomenon in play. What could this predominant phenomenon be? To decipher it, we realized very simple experiments in monitoring the long time behaviour of a number of chemostats using various input substrate concentrations. Recall that the mathematical analysis of chemostat models tells us that the steady state concentration of substrate should not be dependent on the input substrate concentration if the biomass follows a Monod's growth rate function while it should if the growth is more likely density-dependent, *cf.* Krichen *et al.*, 2017. The results confirmed that if density-dependent growth-rate can indeed result from a high concentration of biomass, it can also result from the structuration of the biomass into flocs or in any other kind of biomass structuration limiting its mobility. In particular, the effect was all the more marked as the biomass was structured. For instance, they were very density-dependent in the presence of filamentous bacteria, structuring the whole medium into *a kind of glue*

immobilizing most living cells. This results are likely similar to the numerical results that were put forward in Harmand and Godon, 2007 in which a model was used to simulate the cascade of fixed bed bioreactors: considering immobilized biomass it was shown that a clear density-dependence appeared – which was robust with respect to model parameter values. In addition, even if he probably took care about experimental conditions, it should be noticed that in his experiments, as recalled hereabove, Contois used *Aerobacter aerogenes*, catalogued as immobile bacteria. As the biomass structures itself in the medium, the density-dependent effect could appear not predominantly due to the density but rather to the way it is precisely structured. This could explain why, while Contois used less dense media, he could better describe his experiments with density-dependent functions. All these evidences let us suggest that more than the biomass density in itself, the way it structures into the medium – notably with respect to cell mobility – could play a more important role in the occurrence of density-dependence. Obviously, such structuration of the biomass enters in the framework of *complexity* we tried to define earlier.

4. CONCLUSIONS

For a long time, researchers try to characterize the determinants of the growth of microorganisms, and in particular their dependence with respect to the available essential resources. Monod has been the first to hypothesize that this growth is, before all, an increasing saturated function of the main limiting substrate concentration. Contois assumed that the growth is not only a function of the substrate but also of the biomass density-itself, and thus the name «density-dependent». The intuitive explanation is that at high concentration of biomass, the active biomass is only a fraction of the total – because it becomes difficult for the remaining fraction to access the substrate. Thus, one could have expected to observe that Monod and Contois worked with significantly different biomass concentrations in their experiments. However, re-examining Monod and Contois experiments, it is obviously not the case and μ Contois seems to have worked with smaller concentrations of biomass than Monod. In this paper, we advance another phenomenon than the density in itself for density-dependent phenomenon to appear: the structuration of biomass – and notably the loss or lack of its mobility. For sure, this question leads us to revisit modeling of

density-dependent experiments to more clearly link biomass mobility properties to density-dependent growth rates and opens new experimental perspectives in deciphering the «engines» of density-dependence.

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